

CLAIMS

- 1.- Enzyme product of plant origin designated NPPase, characterized in that its sequence contains at least one of the polypeptide fragments represented by SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8; SEQ ID NO: 9; SEQ ID NO: 10; SEQ ID NO: 11; SEQ ID NO: 12; SEQ ID NO: 13; SEQ ID NO: 14; SEQ ID NO: 15; SEQ ID NO: 16 and SEQ ID NO: 17 and shows phosphodiesterase activity.
- 2.- Enzyme product designated NPPase, according to claim 1 characterised by having a amino acid sequence deduced from a cDNA selected among SEQ ID NO: 20 or SEQ ID NO: 22.
- 3.- Enzyme product, according to claim 2 characterized in that it contains a sequence represented by SEQ ID NO: 21.
- 4.- Enzyme product, according to claim 2 characterized in that it contains a sequence represented by SEQ ID NO: 23.
- 5.- Enzyme product according to any of the claims 1 to 4, characterized in that catalyses the hydrolysis of nucleotide sugars in equimolar conditions to sugar-phosphate and the corresponding nucleoside monophosphate, does not hydrolyse molecules with phosphomonoester bonds, is able to hydrolyse bis-PNPP, is inhibited by molybdate, arsenate and phosphorylated molecules, its activity is not affected by reducing and chelating agents that are inhibitors of phosphodiesterases, it is sensitive to slightly basic pH and is very stable at pH between 4 and 7.5, can be glycosylated, which makes it resistant to ionic detergents of the SDS type and to the action of proteases, and recognizes, in addition to nucleotide sugars, other small molecules that possess phosphodiester and phosphosulphate bonds.
- 6.- Enzyme product as claimed in any of the claims 1 to 5 characterized in that it does not hydrolyse, among others, G1P, G6P, AMP, 3-phosphoglycerate, AMPc, nor nucleic acids.

7.- Enzyme product as claimed in either one of the claims 1 and 6, characterized in that it does not require as effectors, among other divalent cations, magnesium, manganese or cobalt.

8.- Enzyme product as claimed in any one of the claims 1 to 7, 5 characterized in that it is inhibited by orthophosphate, inorganic pyrophosphate, and phosphate esters such as, among others, AMP, ADP, ATP, or 3-phosphoglycerate.

9.- Enzyme product as claimed in any one of the claims 1 to 8, characterized in that its activity is not affected by, among others, β -mercaptoethanol, EDTA, reduced cysteine or ascorbate. 10

10.- Enzyme product as claimed in any one of the claims 1 to 9, characterized in that it is resistant to, among others, Proteinase K or Pronase.

11.- Enzyme product as claimed in any one of the claims 1 to 10, characterized in that it recognizes as substrates, among others, ADPG, UDPG, 15 GDP-glucose, ADP-mannose, APS, PAPS or bis-PNPP, the preferred substrate being ADPG.

12.- Enzyme product as claimed in any one of the claims 1 to 11, characterized in that it is resistant to a temperature of 65°C for 30 minutes, and in 20 that it has an apparent molecular weight determined by gel filtration around 70 and 270 kDa for the monomeric and homopolymeric isoform respectively, as well as displaying a K_{eq}' of the reaction of 110, its $\Delta G'$ being -2.9 kcal/mol, and its K_m for ADPG being 0.5 mMolar.

13.- Enzyme product as claimed in any one of the claims 1 to 12, characterized in that it was isolated from any plant species.

25 14.- Method of obtaining an enzyme product of plant origin with nucleotide sugar pyrophosphatase/phospho-diesterase activity (NPPase) in its soluble isoform, having an amino acid sequence that contains at least one of the polypeptide fragments represented by SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8; SEQ ID NO: 9; SEQ ID NO: 10; SEQ ID NO: 11; SEQ ID NO: 12; SEQ ID NO: 13; SEQ ID NO: 14; SEQ ID NO: 15; SEQ ID NO: 16 and SEQ ID NO: 30

17, characterized in that the material of plant origin is submitted to extraction of the protein fraction by a buffer, filtration of the extract, followed by a method of purification by successive centrifugations and precipitations, with adjustments both of the pH and of the ionic strength of the medium, preferably including 5 heating of the protein above 60°C and cooling in ice, and purification by gel filtration, isoelectric focusing, denaturing-gel electrophoresis, or other equivalent means of purification of proteins extracted from plant tissues.

15.- Method as claimed in claim 14 comprising the following steps: (1) homogenization of the plant tissue with an extraction buffer, type Mes 50 mM pH 10 6, EDTA 1 mM, DTT 2 mM, (2) filtration, (3) ultracentrifugation at 100 000 g, (4) precipitation of the proteins of the supernatant in ammonium sulphate, (5) resuspension of the precipitate in buffer of pH 4.2, (6) heating for at least 15 minutes at a temperature between 60 and 65°C, followed by cooling in ice, (7) centrifugation at 30 000 g, (8) concentration of the protein of the supernatant by 15 precipitation in ammonium sulphate and resuspension at pH 6, and (9) purification by gel-filtration chromatography, isoelectric focusing and denaturing-gel electrophoresis.

16.- Primers represented by SEQ ID NO: 18, SEQ ID NO: 19 or SEQ ID NO: 24.

20 17.- Use of primers represented by SEQ ID NO: 18, SEQ ID NO: 19 or SEQ ID NO: 24 together with an mRNA from leaves of rice or barley for obtaining, by RT-PCR, a cDNA which, after being used as a probe on cDNA libraries of leaves of rice and barley, permits the isolation of cDNA's whose sequences are represented by SEQ ID NO: 20 and SEQ ID NO: 22, respectively.

25 18.- cDNA represented by SEQ ID NO: 20 that codes for an enzyme product with NPPase activity.

19.- Use of primers represented by SEQ ID NO: 18, SEQ ID NO: 19 or SEQ ID NO: 24 together with an mRNA from barley leaves for obtaining, by RT-PCR, a cDNA which, after being used as a probe on cDNA libraries of barley 30 leaves, permits the isolation of cDNA whose sequence is represented by SEQ ID NO: 22.

20.- cDNA represented by SEQ ID NO: 22 that codes for an enzyme product with NPPase activity.

21.- Use of the enzyme product of claims 1 to 13 in the preparation of assay devices and/or compositions for application in the determination of 5 nucleoside diphosphate sugars.

22.- An assay device for the determination of nucleoside diphosphate sugars, characterized in that it includes the enzyme product of claims 3 to 13 and 19 or 20 in such a way that the determination is based on the sugar-1-phosphate released during the reaction catalysed by NPPase.

10 23.- The assay device as claimed in claim 22, characterized in that the determination is based on the glucose-1-phosphate released, which is submitted to the enzyme phosphoglucomutase to produce glucose-6-phosphate, which in its turn is submitted to a coupled reaction with NAD^+ and NADP^+ , by the action of the enzyme glucose-6-phosphate dehydrogenase, obtaining 6-phosphogluconate 15 and NADH or NADPH, either of which can be determined by conventional spectrophotometric methods or methods of some other kind.

20 24.- An assay device for the determination of nucleoside diphosphate sugars, characterized in that it includes the enzyme product of claims 1 to 13, in such a way that the determination is based on the nucleoside monophosphate produced during the reaction catalysed by NPPase.

25 25.- The assay device as claimed in claim 24, characterized in that the determination is based on the nucleoside monophosphate, which is able to release orthophosphate, in addition to the corresponding base, by the action of an enzyme such as 5'-nucleotidase, the orthophosphate being easily determined by conventional methods, for example colorimetric methods.

30 26.- The assay device as claimed in any of the claims 22 to 25, characterized in that the determination is based on the fact that the sugar-1-phosphate and the nucleoside-monophosphate are able to release orthophosphate by the action of an enzyme such as alkaline phosphatase or 5'-nucleotidase, the orthophosphate being easily determined by conventional methods, for example colorimetric methods.

27.- Use of the enzyme product of claims 1 to 13 in the preparation of assay devices and/or compositions for application in the determination of the presence of 3'-phospho-adenosine 5'-phosphosulphate (PAPS) and adenosine 5'phosphosulphate (APS).

5 28.- Use of the primers of claims 16 and the cDNA's of claims 18 or 20 in the production of transgenic plants that express or overexpress the cDNA that codes for NPPase.

10 29.- Method of production of transgenic plants that express or overexpress the gene that codes for NPPase, characterized in that a transformation vector is used that contains a plasmid that includes the cDNA represented by SEQ ID NO: 20 of the gene of the said NPPase.

15 30.- Method of production of transgenic plants that express or overexpress the gene that codes for NPPase, characterized in that it uses a transformation vector that contains a plasmid that includes the cDNA represented by SEQ ID NO: 22 of the gene of the said NPPase.

31.- A method of production of transgenic plants as claimed in claim 29 or 30, characterized in that the transformation vector is *Agrobacterium tumefaciens* CECT 5799.

20 32.- Transgenic plants obtainable by the method as claimed in claims 29 to 31, characterized in that they express or overexpress the enzyme product of claims 1 to 13 and have a reduced content of starch and/or of cell-wall polysaccharides and are resistant to high temperatures and to high salinity.

25 33.- An assay device for the determination of sulphonucleotides, characterized in that it includes the enzyme product of claims 1 to 13 in such a way that the determination is based on the sulphate that is released.